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THE BIOLOGICAL CLASSIFICATION OF INFLUENZA BACILLI

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Since the discovery of *B. influenzæ* medical literature has [50] become burdened with discussions about its relationship to epidemic and sporadic influenza, pneumonia, pertussis, diphtheria, measles, scarlet fever, conjunctivitis, and meningitis, whether it is really hemoglobinophilic, and whether all strains are alike morphologically, culturally and immunologically. It is very interesting reading, but, after all, the answer to many questions seems no nearer than it was almost 30 years ago. A brief review is necessary to show the exact status of *B. influenzæ* and the difficulties presented in undertaking a study of this kind.

Pfeiffer[1] described a small Gram-negative bacillus in smears from the pharynx and the sputum of influenza patients. Later he cultivated the bacillus, finding hemoglobin essential for its growth on artificial media, and further characterized it as non-motile, aërobic, not found in the blood of influenza patients, and not very pathogenic for animals. Afterwards he found in three cases of bronchopneumonia following diphtheria in children a bacillus in every respect the same as the one spoken of as the true influenza bacillus, with the exception that it was larger and developed many thread-like forms. This organism he called the pseudo-influenza bacillus. The differentiation was made on morphology alone. Wolff,[2] in 1903 stated that Pfeiffer believed all influenza bacilli were the same, and that his original idea of a pseudo-influenza bacillus was incorrect.

[50] Cantani [3] thought that the pseudo-influenza bacillus and the true one were identical, and that neither of them were hemoglobinophilic, as they could be grown on media, enriched with spermatic fluid, which did not give the spectroscopic band of hemoglobin. Ghon and Preyss [4] considered hemoglobin necessary even though it were present in such small quantities that
[51] it failed to give a band with the spectroscope unless hydrazin were added. Neisser [5] was able to grow an influenza-like bacillus, isolated from a case of purulent conjunctivitis, on plain agar for 20 generations in symbiosis with a xerosis bacillus. Davis [6] thought hemoglobin, acting as a catalytic agent, was necessary for growth, and showed that a very small amount was required (1 part in 180,000 parts of medium). According to him, growth will take place in the presence of coagulated hemoglobin, but if the hemoglobin be broken up by excessive heating into hematin and globin, no growth occurs.

The difficulties which arise in the exact identification of an influenza bacillus have caused many mistakes in the past and probably will continue to cause them. Spengler,[7] Luzzatto[8] and Jochmann and Krause [9] have described at different times a bacillus which they considered the cause of pertussis. It is now believed they were dealing with the group of influenza bacilli and not the organism later described by Bordet and Gengou.

Gram-negative hemoglobinophilic bacilli have been recovered from different parts of the human body, also from animals, under a variety of conditions. Rosenthal [10] considered them ordinary members of the mouth flora without pathogenic significance. Auerbach,[11] Davis,[12] and many others have isolated them from the throats of patients with measles, pertussis, diphtheria and scarlet fever. Klieneberger [13] described a pseudo-influenza bacillus which he isolated from pus in a gall-bladder. Cohn [14] recovered *B. influenzæ* from a case of acute urethritis. It is generally known that these organisms may cause sinusitis, pneumonia, septicemia, endocarditis, arthritis, otitis media, and meningitis. Davis [15] isolated from the urine of three patients a small, Gram-negative, hemolytic, anaërobic, hemoglobinophilic bacillus. Moon [16] recovered a

small, Gram-negative anaërobic, hemoglobinophilic bacillus from a chronic ethmoid infection. Friedberger,[17] working in Pfeiffer's clinic, isolated from the preputial secretions of a dog a small, Gram-negative, non-motile, hemoglobinophilic bacillus. Wolff,[2] working in the same clinic, isolated from the lungs of a rat a bacillus that remained exactly like *B. influenzæ* for three months, after which time it acquired the property of growing on ordinary media. Pritchett and Stillman[18] have recovered from throat cultures a Gram-negative, non-motile, aërobic, hemolytic, hemoglobinophilic bacillus which makes milk alkaline (growth takes place when a little blood is added to the milk). [51]

In 1905 Wollstein,[19] working on pertussis, isolated bacilli considered as belonging to the influenza group, but from which they could be differentiated by agglutination and absorption tests. In 1906,[20] not feeling so positive about this difference, she says: "The similarity of cultural characteristics of all the influenza bacilli has been emphasized by Neisser, and my experience with the agglutination reactions leads me to regard all strains as belonging to one family."

Meunier[21] (1897) reported 10 cases of broncho-pneumonia in young children caused by *B. influenzæ*. In several instances the bacillus was recovered from the blood both before and after death. At this time, contrary to Pfeiffer's statement, it was noted that these organisms were highly pathogenic for rabbits. Slawyk,[22] in 1899, isolated an influenza bacillus from the blood and spinal fluid of a child with meningitis. This organism was seen by Pfeiffer who agreed that it was *B. influenzæ*. Cohen[23] (1909) recovered from the spinal fluid and blood of patients with meningitis an organism similar to the influenza bacillus, except that it caused septicemia in rabbits, and because of this difference and evidence obtained by protection experiments, he was inclined to believe that the two organisms were not the same. Thursfield,[24] in 1910, reported two cases of *B. influenzæ* septicemia without meningitis. He agreed with Cohen and concluded by saying, "Organisms hitherto described as *B. influenzæ* are not all identical, but like the coli-typhoid family, belong to a group the various members of which possess very different pathogenic powers." Wollstein,[25]

[51] in 1911, working with respiratory and meningeal strains, considered them identical, and varying only in virulence. Davis,[26] in 1911, found no difference in the virulence of respiratory and meningeal strains. Wollstein,[27] in 1915, still regarded all influenza bacilli as more or less identical regardless of their own origin or virulence. Davis,[12] in 1915, suggested dividing them into two groups, one showing the phenomenon of symbiosis, the other lacking it. Williams,[28] in 1919, working with a number of strains reported a few crosses by agglutination, but none by absorption tests. Huntoon and Hannum,[29] in 1919, say, "We have found no strains among our collection which do not show relationship either directly or indirectly through absorptions." Gay and Harris,[30] in 1919, found in their serological work on influenza evidence that influenza bacilli probably could be divided into groups.

In spite of the vast amount of work done, very little is known about *B. influenzæ* and its biological activities. At present it is described as a small, Gram-negative, aërobic, nonmotile, hemoglobinophilic bacillus. For all that is known, there may be a number of different kinds of bacilli answering that description, or there may be only one. In reality there is one true *B. influenzæ* existing in name only, and that is the first one grown and described by Pfeiffer, as he did not and could not prove any of the subsequent strains to be identical with the first.

Jordan[31] has made the best contribution lately to our knowledge of influenza bacilli by showing that 10 of 13 strains formed indol. Owing to this discovery a study of the cultural characteristics of different strains of *B. influenzæ* was undertaken. The work has not been completed, and what follows is merely a preliminary report made at this time with the hope that others will become interested and assist in the solution of the problem.

The bacilli for study were obtained from normal throats since the epidemic, 32 strains; from influenza meningitis, 5 strains (4 coming from Dr. Howland's clinic this year and 1 isolated by Dr. Wollstein in 1917); and from cases of epidemic influenza, 14 strains (supplied by Drs. Parker, Wollstein and Stillman). Two strains of *B. pertussis*, one from

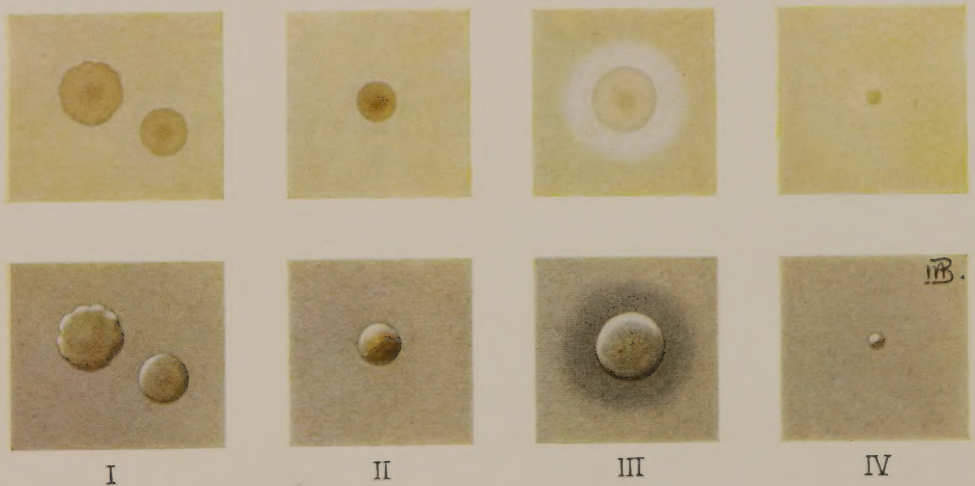

Fig. 1.

Dr. W. W. Ford, the other from the N. Y. Board of Health [51]
were studied at the same time for comparison. In isolating the [52]
organisms from normal throats, all Gram-negative, aërobic, hemoglobinophilic bacilli were included in the series regardless of morphology and the kind of colony formed, since these latter vary so much under different conditions that they are unreliable criteria.

In a study of this kind it is necessary to be able to obtain at all times a vigorous growth of the organisms both on solid and in liquid media and describe their colony formation, morphology and biological activities under as favorable conditions as possible. Meat-infusion agar and broth, Ph 7.5, with from 1 to 2 per cent rabbit blood, furnish good media for growth and to which other ingredients may be added for study.

All the strain are hemoglobinophilic after at least two months of artificial cultivation, except in a few instances, in which they were grown on hemoglobin-free media in symbiosis with other bacteria for several generations. They are Gram-negative, non-motile, do not liquefy blood-gelatin, do not ferment glucose, lactose, saccharose, maltose, mannite, salicin, inulin, glycerol and xylose in one week, and, most of them, including the three hemolytic ones, have at some time shown the phenomenon of symbiosis. Three strains hemolyse rabbit blood both in liquid and in solid media.

In working with the morphology and colony formation, it is necessary to grow the various strains side by side, generation after generation, on exactly the same medium, and then it is possible to notice in a general way differences between them. Fig. 1 (magnification 8) shows colonies of four different organisms 36 hours old, grown under the conditions named above, the upper row as seen by direct light, the lower by top light. No. 1 forms indol, No. 2 forms amylase, No. 3 is hemolytic, No. 4 is *B. pertussis*. Nos. 1 and 3 are moist, become very granular and dark in the center, and when one to five days old put out daughter colonies. No. 2 is tough, holds its shape, and puts out very few daughter colonies. All pit and turn the medium a dirty brownish color. All are tan-colored after 48 hours. Older observers attributed this color

[52] to the hemoglobin in the medium, but this is unlikely as it occurs when they are grown on hemoglobin-free media in symbiosis with other bacteria. Figs. 2 and 3 (magnification 20) are copies from Pfeiffer [1] and Grassberger [32] showing the type of colonies with which they were working.

When a solid medium is inoculated with the various strains, there is seen a distinct and constant difference between the edges formed. Cultures of *B. pertussis* (Fig. 4) have elevated, smooth edges; some of the influenza bacilli (Figs. 5 and 6), elevated, lobate edges; others (Figs. 7 and 8), slightly elevated, finely irregular edges; and others which grow very delicately have no sharply defined edges. (See lower right corner of Fig. 9 which also shows two other types of cultures mentioned above, and finally *B. pertussis* in the upper right corner). Smears from cultures with the lobate edges usually show the typical small bipolar-staining bacilli called the true influenza bacillus, whereas smears from the ones with finely irregular edges and no sharply defined edges usually show large, more easily stained bacilli, bizarre shapes, and thread-like forms. This difference is well shown by copies of Pfeiffer's [1] true (Fig. 10) and his pseudo-influenza (Fig. 11) bacilli.

One of the first things noticed when working with influenza bacilli was that some strains both on solid and in liquid media soon developed a perfectly characteristic fresh fecal odor. Later, when cultures of the different strains were tested for indol formation, it was found that the ones which produced this odor gave positive tests for indol when the ether extracts were layered with Ehrlich's reagent. Cultures have been distilled and ether extracts of the water-clear distillate gave the same results as were obtained before distillation. Repeated tests have been made on the various strains grown from two days to two weeks, and indol has been produced constantly by some, and not, just as constantly, by others. Thirty of the 51 strains form indol and the five meningeal ones are in the positive group. The spinal fluids of patients with influenza meningitis do not give a positive test when first drawn, but, if a tube of it be placed in an incubator for from 12 to 24

hours, a definite test is obtained. The fluid from a meningo- [52]
coccus meningitis gave a negative test when thus treated.

The ability of the various influenza bacilli to form amylase has been studied in the following manner. Defibrinated rabbit blood was added to melted meat-infusion agar which was kept at 95° C. long enough to destroy the amylase present in the blood. Then to 100 c. c. of this mixture were added from 10 to 15 c. c. of a 2 per cent sterile soluble starch solution. Plates were poured, allowed to cool and inoculated at various points with different influenza bacilli, and after from 3 to 5 days incubations were covered with a weak Lugol's solution. If the starch were not split, the medium was a dark blue up to the edge of the cultures, but if it were changed, the iodine reaction was absent around them, a colorless zone of from 4 to 8 mm. in width being left. A positive and a negative reaction are shown in Fig. 12. Nine strains produce amylase in small amounts, and none of these produce indol.

Tubes of potassium nitrate blood-broth were inoculated with influenza bacilli, incubated for 5 days, and then tested with the sulphanilic acid and naphthylamin reagent for nitrites. Thirty-three of the 51 strains have at some time given a positive reaction. Some always reduce the nitrates, others do so irregularly, while still others have never done so. This characteristic is displayed by some indol formers, by some amylase formers and by two of the three hemolytic ones. Further work may show that all can reduce nitrates under the proper conditions, making this a common characteristic of the whole group, just as hemoglobin is essential for their growth.

Flasks of 100 c. c. of fat-free milk with brom-cresol purple for an indicator, and flasks of 100 c. c. meat-infusion broth with the same indicator were autoclaved separately. (If autoclaved together a precipitate forms which interferes.) After cooling, equal quantities of the milk and the broth were mixed and from 1 to 2 per cent rabbit blood was added. This is a good medium for the growth of influenza bacilli, and accurate readings can be made when an uninoculated tube is [53]
kept for a control. When these tubes of blood-broth-milk were inoculated and incubated, it was obvious very soon that all influenza bacilli are not the same culturally, as some,

[53] within 48 hours, made the milk slightly but definitely acid, others slightly but definitely alkaline, while still others gave doubtful results. This difference persisted for a week. Readings made at intervals longer than a week cannot be given at this time. The source of the acid is unknown, since lactose is not fermented.

DISCUSSION

Many people have never felt absolutely certain that the differences between *B. pertussis* and *B. influenzæ* were sharp enough to be beyond doubt in spite of the serological proof. *B. pertussis*, after a period of artificial cultivation, can be grown on plain media, forms no indol, no nitrites and makes milk very alkaline. Some of the influenza bacilli also form no indol and no nitrites, but none of them has ever made milk nearly as alkaline as *B. pertussis*.

Time alone will tell whether these cultural characteristics will be constant. While there are differences in the biological activities of the various strains of *B. influenzæ*, at the same time there are definite groups, the individual members of which are similar culturally. Only one group will be discussed at this time. It consists of 10 strains five from the spinal fluid of patients with influenza meningitis, two epidemic strains from New York, and three from normal throats. The growth and morphology of these are similar, all form indol, all reduce nitrates to nitrites, and make blood-broth-milk slightly acid within 48 hours.

Whether the strains of large bacilli that are amylase formers, or the hemolytic ones, should be included in this big influenza group is a question that will have to be decided. The nine amylase formers and the three hemolytic strains have characteristics in common with the big group, as shown by certain ones forming nitrites and by one of the hemolytic strains forming both indol and nitrites. Possibly this is a big group of organisms, like the streptococci, which have been divided into hemolytic and non-hemolytic strains, and further subdivided by cultural characteristics and serological tests. Possibly the group can be compared with the Gram-negative diplococci, Meningococcus, Parameningococci, Micro-

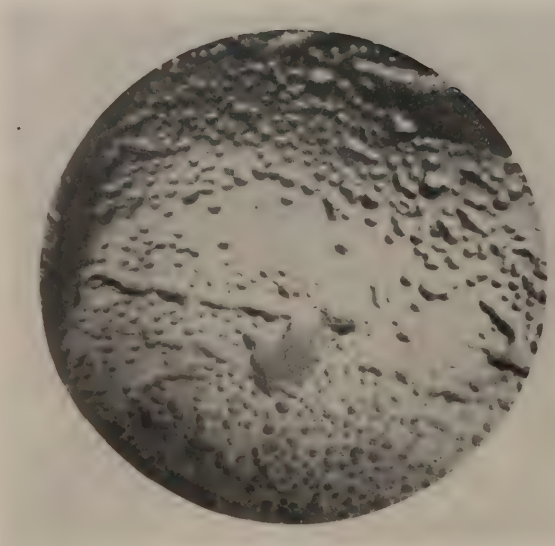

FIG. 2.

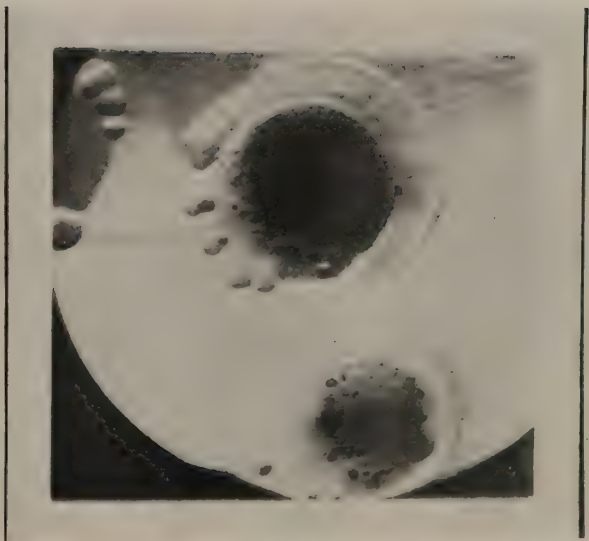

FIG. 3.

FIG. 12.

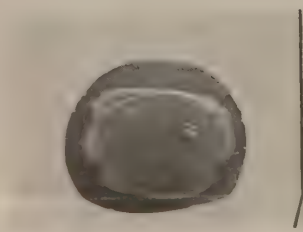

FIG. 4.

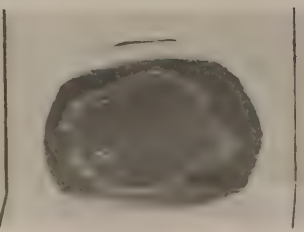

FIG. 5.

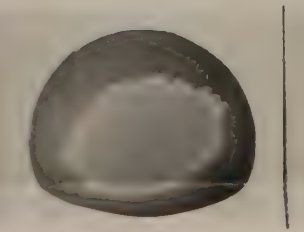

FIG. 6.

FIG. 7.

FIG. 8.

Fig. 9.

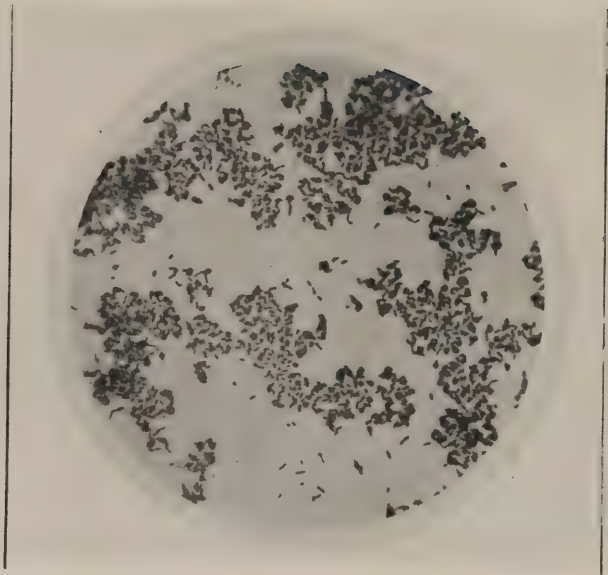

Fig. 10.

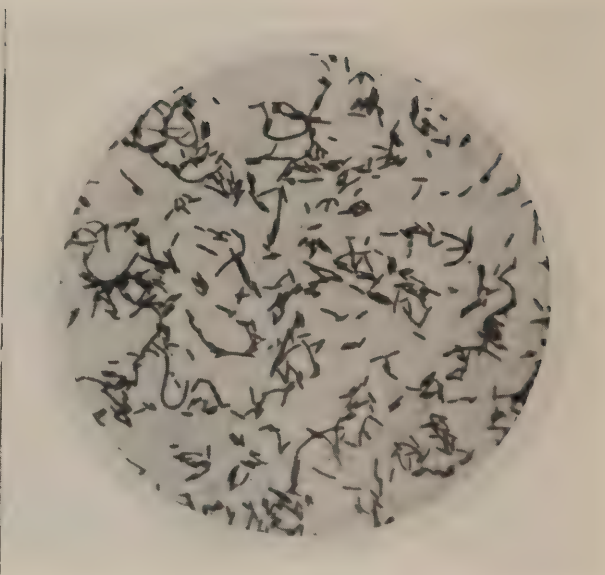

Fig. 11.

coccus catarrhalis, Micrococcus flavus, Micrococcus pharyngis siccus, Gonococcus and others. [53]

CONCLUSIONS

1. The Gram-negative, non-motile, hemoglobinophilic bacilli can be classified biologically by reactions which admit of subdivisions of the group.

2. In working with a suspected *B. influenzæ,* the following routine should be followed:

(a) Determination of hemoglobinophilic qualities.
(b) Colony formation.
(c) Hemolytic test.
(d) Gram stain.
(e) Morphology.
(f) Motility.
(g) Indol formation.
(h) Reduction of nitrates to nitrites.
(i) Amylase formation.
(j) Reaction in blood-broth-milk.

3. *B. pertussis* can be differentiated from the group of *B. influenzæ* by cultural characteristics.

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